Searches Among Mycobacterial Cultures for Antileprosy Vaccines

CHARLES C. SHEPARD,* ROSALIND VAN LANDINGHAM, AND LAURA L. WALKER

Leprosy and Rickettsia Branch, Center for Disease Control, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta, Georgia 30333

All mycobacteria species share some antigens, so there may be cultivable mycobacterial cultures that can provide vaccine protection against leprosy. Vaccine protection against *Mycobacterium leprae* infections in mice has been demonstrated for *M. leprae* itself, as living or heat-killed suspensions, and for *Mycobacterium bovis* (BCG), as living suspensions. Results are reported here with 17 other cultures. The mycobacterial suspensions were injected intradermally, and the mice were challenged in the footpad with infectious suspensions of *M. leprae*. In two experiments the mice were also challenged by footpad injections of 10^7 heat-killed *M. leprae* so the footpad enlargement could be measured. That some mycobacterial suspensions were immunogenic for some of their own antigens was suggested by reactions at the vaccine site and enlargement of the regional lymph nodes. Some mycobacterial suspensions also stimulated footpad enlargement on challenge by homologous suspensions or by challenge with *M. leprae* suspensions. Consistent protection against infectious challenge with *M. leprae* was observed only with BCG and *M. leprae*, however.

Two general approaches are being taken in the search for a leprosy vaccine. (i) The use of *Mycobacterium leprae* itself, which may be practical because larger numbers of *M. leprae* can now be produced in armadillos (4) and because *M. leprae*, in contrast to many other mycobacteria, remains immunogenic when killed (16). Some of the procedures used to purify *M. leprae* from the infected armadillo tissues are harmful to immunogenicity or give poor yields (C. C. Shepard, P. Draper, R. J. W. Rees, and C. Lowe, Br. J. Exp. Pathol., in press; C. C. Shepard, F. Minagawa, R. Van LANDINGHAM, and L. L. Walker, submitted for publication), but these technical problems can probably be solved. Difficulties with supply of *M. leprae* for large-scale production of vaccine seem likely to remain, however, because infected armadillos are inconvenient to maintain and because 1 to 2 years are needed after inoculation before full-blown infections develop. (ii) The use of cultivable mycobacteria. All mycobacteria have some antigenic relationships, and it may be that a culture can be found that will provide useful protection against *M. leprae*. Supply and purification of the vaccine would thus be simplified. In this paper we present results with 17 selected mycobacterial cultures as vaccines against *M. leprae*.

In earlier work we found that *M. duvali* suspensions provided no protection against *M. leprae* infection (15). This culture had been found to be efficient in stimulating lymphocyte trans-

formation to *M. leprae* in rabbits (4); the immunizing antigen was given in Freund incomplete adjuvant.

In still earlier work we found evidence that heat-killed *M. tuberculosis* (H37Rv) and a strain of the *M. avium-M. intracellulare* complex (QT) stimulated protection against *M. leprae* infection (13). In that work, however, the methods for measuring vaccine activity were less quantitative than those used now.

MATERIALS AND METHODS

Except as noted, the mycobacterial cultures were maintained on Loewenstein-Jensen medium. For the suspensions used as vaccines, cultures were grown in Tween-albumin medium (Tb-Tween, Difco Laboratories, Detroit, Mich.), harvested as young cultures, washed once in phosphate-buffered saline (pH 7.3) with 0.05% Tween 80, and preserved at -60°C . The final concentration for use in the mice was judged by the cell pack in Hopkins tubes centrifuged for 60 min at $2,200 \times g$. The amount injected per mouse was $0.1 \mu\text{l}$ of cell pack, which is equivalent to $10 \mu\text{g}$ (dry weight), 10^7 bacilli, and, in the case of the Rosenthal strain of *M. bovis* BCG, 10^6 colony-forming units. To save space the cultures are listed in each experiment together with the rationale for their selection.

The *M. leprae* suspensions were purified from experimentally infected armadillo liver by suspension, 5-min trypsin treatment (14), and the aqueous two-phase (dextran-polyethylene glycol) separation that Draper applied to *M. leprae* (11). All *M. leprae* suspensions were heated to 100°C for 30 min in their final concentrations.

The cord factor, used in experiment 3, was generously provided by E. Lederer, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France. It had been prepared from the Peurois strain of *M. tuberculosis*.

Female CFW mice, raised at the Center for Disease Control, were used at an age of 8 to 12 weeks. For immunization, the mycobacterial suspensions were given in a volume of 0.01 ml intradermally in the flank near (but not over) the inguinal lymph node (15). The sizes of the inguinal lymph node and the reactions at the site were measured at intervals (15).

For footpad enlargement (FPE) the thickness of the foot was measured with dial calipers just before and 1, 2, and 3 days after the injection of challenge antigen, which was given 28 days after the immunizing antigen. The challenge antigens were mycobacterial suspensions (heated to 100°C for 30 min) containing 10^7 *M. leprae* or 0.1 μ l of the other cultures in the 0.03 ml injected into the footpad. For corrected FPE, the average enlargement in unimmunized control mice was subtracted.

For infections challenge, 5,000 *M. leprae* in mouse passage were injected into the right hind footpad 28 days after vaccination. The *M. leprae* growth curve in normal mice was monitored by counts of pools of four mice every 4 weeks until the curve passed $10^{6.0}$ per mouse, a signal that the plateau level had been reached. Counts were then carried out on eight individual mice from each vaccinated group and from each of the two control groups. These harvests were completed within 1 working week. The counts were repeated 90 days later. Protection equals $\log_{10}$ (average harvest in controls) minus $\log_{10}$ (average harvest in test group).

The significance of differences between groups was assessed by the two-sample rank test. The values shown are for a two-tailed test.

RESULTS

In experiment 1, the following cultures were tested (Fig. 1 and 2): (i) *M. bovis* BCG Rosenthal the culture we have usually used in previous work and which originated from a BCG vaccine prepared at the Chicago Research Institute; (ii) *M. bovis* BCG Trudeau, purchased from the Trudeau Institute, Saranac Lake, N.Y., and received as a frozen suspension on dry ice and kept at -60°C until used (mycobacterial content, as judged by turbidity, was less than that of BCG Rosenthal); (iii) *M. chelonae* strain 58; (iv) *M. diernhoferi* 295; (v) *M. nonchromogenicum* 269 (from NCTC 10424); (vi) *M. nonchromogenicum* 393; (vii) *M. nonchromogenicum* 1481 (from ATCC 19530); (viii) *M. phlei* 3 (from NCTC 8156); (ix) *M. smegmatis* 1 (from NCTC 333); (x) *M. vaccae* 296 (rough variant); (xi) *M. vaccae* 426 (from ATCC 15483); (xii) *M. vaccae* 1526 (from ATCC 19250). Cultures iii to vi and viii to xi were obtained from J. Stanford; cultures vii and xii were from the Trudeau Institute mycobacterial collection.

Paul et al. (10) found in leprosy patients in east Africa that soluble extracts of *M. nonchromogenicum* and *M. vaccae* gave skin test results that resembled those expected from *M. leprae*, namely, a low rate of positivity in lepromatous patients and a distinctly higher rate in tuberculoid patients. Their extracts were prepared by sonicating whole organisms and then filtering the $70,000 \times g$ supernatant. Stanford and Rook found by immunodiffusion analysis the extracts of *M. vaccae* resembled those of *M. leprae*; the extracts of only these two species lacked the antigens of both fast-growing and slow-growing groups (18). Thus, the *M. nonchromogenicum* and *M. vaccae* cultures were included in the experiment to see if the relationship might extend to vaccine protection. The cultures of *M. chelonae*, *M. diernhoferi*, *M. smegmatis*, and *M. phlei* were included as negative controls, and BCG was included as a positive control.

The size of the lymph nodes regional to the vaccine site is shown in Fig. 1 for two illustrative intervals. The two BCG vaccines caused highly significant ($P < 0.001$) enlargement at 36 and 179 days. The only other cultures that produced lymph node enlargement for the group with a P value of < 0.01 were *M. diernhoferi* and *M. vaccae* at 36 days and *M. nonchromogenicum* 1481 at 179 days.

The results of the harvests are shown in Fig. 2. The only cultures that provided highly signif-

FIG. 1. Lymph node size in mice vaccinated with various mycobacterial cultures in experiment 1. BCG, BCG strain of *M. bovis*; CHE, *M. chelonae*; DIE, *M. diernhoferi*; NC, *M. nonchromogenicum*; PHL, *M. phlei*; SME, *M. smegmatis*; and VAC, *M. vaccae*. Symbols: ●, individual mouse; □, group average. P^* values: 0, > 0.05 ; 1, 0.01 to 0.05; 2, 0.001 to 0.009; 3, < 0.001 .

FIG. 2. Harvests of *M. leprae* from mice vaccinated with various mycobacterial cultures in experiment 1. (abbreviations as in Fig. 1). The challenge was with 5,000 infectious *M. leprae* 28 days after vaccination. *P** values: 0, >0.05; 1, 0.01 to 0.05; 2, 0.001 to 0.009; 3, <0.001

icant protection were the two BCG cultures. No other vaccines afforded protection that was much more than borderline. A possible exception is *M. diernhoferi*, for which the *P* values were <0.05 in both harvests. As noted above, the mice receiving this vaccine had had slightly enlarged regional lymph nodes at 36 days.

In experiment 2 the following suspensions were tested. (i) *Mycobacterium* sp. 100616—Nakamura found this culture, even when heat-killed, to produce prominent reactions when injected into the footpad (9). R. A. Good of the Mycobacterial Branch at the Center for Disease Control found it to be a rapidly growing species that was neither *M. fortuitum* nor *M. chelonae*. (ii) *M. tuberculosis* H37Ra—We had previously tried a suspension sent to us by A. S. and G. P. Youmans as a control for a ribonucleic acid preparation (17). The lack of activity by the culture was surprising, so we tried a suspension prepared under the identical conditions used for the other cultures. (iii) and (iv) *M. simiae* 5135, received as *M. habana*, and *M. simiae* 5138—Collins et al. (3) found these cultures (from the Trudeau Institute mycobacterial collection) to persist well in the popliteal lymph node after being injected into the footpad. (v) and (vi)—*M. nonchromogenicum* R507 and *M. vaccae*—These were cultures from J. Stanford that he thought might be better related to *M. leprae* than the ones tried in experiment 1. (vii) *M.*

bovis BCG (Rosenthal) was included in this experiment. (viii) *M. leprae*—This culture was prepared from infected armadillo liver by suspension, centrifugal washing, 5-min trypsin treatment (14), and the aqueous two-phase (dextran-polyethylene glycol) separation that Draper applied to *M. leprae* (11). The *M. leprae* suspension was heated to 100°C for 30 min; the others were freshly thawed and unheated.

The results of experiment 2 are summarized in Table 1. All cultures except *M. nonchromogenicum* produced greater local reactions than *M. leprae* did. All cultures except *M. tuberculosis* H37Ra and *M. nonchromogenicum* R507 produced enlarged lymph nodes. The *M. simiae* cultures and BCG caused the greatest lymph node enlargement. The lymph nodes were also measured through the intact skin at the time of the 6- and 9-month harvests; the average sizes then were about the same as at 28 days.

Significant corrected FPE with *M. leprae* as challenge antigen was seen for all vaccines except *M. vaccae*. The FPE in the BCG- and *M. leprae*-vaccinated groups was significantly larger, however, than that in any other group. With *Mycobacterium* sp. 100616 and BCG as challenge antigens, the FPE in the homologous group was not significantly greater than that in the *M. leprae*-vaccinated group; the uncorrected FPE was quite large, however.

Significant protection against *M. leprae* infec-

TABLE 1. Results of experiment 2 in which mice were vaccinated intradermally with the live cultures shown and with heat-killed *M. leprae* (ML)^a

Group	Vaccine	Reaction to vaccine				Corrected FPE (48 h)			Protection			
		Site, 28 days		Ln, 28 days		Ag ^{d,e}	0.01 mm	P ^c	6 mo		9 mo	
		mm	P ^b	mm	P ^c				Log ₁₀	P ^c	Log ₁₀	P ^c
A	100616	2.26 ^f	<0.0001	3.84 ^f	<0.02	ML	30.5	<0.01	0.25	NS	0.16	NS
B	H37Ra	1.50	<0.02	3.75	NS	ML	40.5	<0.002	0.20	NS	0.21	NS
C	Habana	2.28	<0.0001	6.13	<0.002	ML	28	<0.002	0.20	NS	0.19	NS
D	Simiae	2.20	<0.0004	4.60	<0.002	ML	40	<0.002	0.05	NS	0.35	NS
E	Nonchrom	0.50	NS	3.88	NS	ML	29.5	<0.002	0.52	<0.10	0.32	NS
F	Vaccae	1.85	<0.001	3.98	<0.02	ML	11	NS	0.41	NS	0.95	<0.002
G	BCG	1.58	<0.001	4.78 ^g	<0.002	ML	66.5	<0.002	0.82	<0.01	0.40	<0.02
H	ML ^e	0.49 ^h		3.90 ^h	<0.01	ML	67.5	<0.002	1.09	<0.002	1.63	<0.002
I+P	Nil	0.00		3.23		ML	(32.5) ⁱ		(6.00) ^j		(5.98) ^j	
J	100616					100616	89.5	<0.002				
L	ML ^e					100616	65.5	<0.002				
N	Nil					100616	(75) ⁱ					
K	BCG					BCG	56	<0.002				
M	ML ^e					BCG	27.5	NS				
O	Nil					BCG	(94.5) ⁱ					

^a The reaction at the vaccination site and the size of the regional lymph node (Ln) are shown at 28 days. The corrected FPE is given for 48 h after challenge at 28 days with 10⁷ heat-killed mycobacteria. Vaccine protection [log (average harvest in controls) - log (average harvest in vaccinated group)] is shown at 6 and 9 months after footpad challenge at 28 days with 5,000 *M. leprae* (viable suspension).

^b Versus controls (groups H, L, and M). NS = *P* > 0.10 (two-tailed test values).

^c Versus control groups (nil).

^d Ag, Challenge antigen.

^e Heated to 100°C for 30 min.

^f Groups A and J.

^g Groups G and K.

^h Groups H, L, and M.

ⁱ Uncorrected value.

^j Average harvest in controls.

tious challenge in both harvests was seen only in the BCG- and *M. leprae*-vaccinated mice. Protection was seen in the *M. vaccae*-vaccinated mice at 9 months but not at 6 months.

Experiment 3 involved a culture of "*Mycobacterium W*" (marked MW) sent by P. Talwar (All India Institute for Medical Research, Delhi, India), who stated that it sensitized mice well even when it was heat killed. It has been suggested as a vaccine candidate on the basis of its skin reactions in leprosy patients (8) and other properties. R. A. Good of the Mycobacterial Branch at the Center for Disease Control identified it as a member of the *M. avium-M. intracellulare-M. scrofulaceum* complex. Live BCG and heat-killed *M. leprae* were included in the experiment as positive controls. The results (Table 2) were as follows. (i) The MW culture, even when heat-killed (MW-H), caused significant local reaction and regional lymph node enlargement at 28 days. Significant lymph node enlargement was not found at the 6- and 9-month harvests, however. (ii) The MW culture also sensitized the mice to produce corrected FPE on challenge with MW-H antigen at +28 days. The uncorrected FPE to MW-H was large, but this evidence also suggests

that the MW preparation was immunogenic. (iii) MW and MW-H did not sensitize the mice to produce significant FPE on challenge with *M. leprae*, however. BCG, as usual, did sensitize the mice to challenge with *M. leprae*. (iv) *M. leprae* did not sensitize the mice to produce the expected amount of homologous FPE in this experiment, for reasons unknown. (v) MW and MW-H did not afford significant protection against infection with *M. leprae*, but BCG and *M. leprae* did. The protection provided by the *M. leprae* vaccine at 6 months was not significant, apparently as a reflection of the low immunogenicity of this preparation of *M. leprae*.

Groups I and J were included to follow up on an earlier experiment in which cord factor had failed to show adjuvant activity (Shepard et al., in press). In most previous work on the adjuvanticity of cord factor, it has been administered as a solution in oil, often emulsified in aqueous media (1, 12), and in our earlier experiment we had emulsified the bacillary suspension in Freund incomplete adjuvant in which the cord factor had been dissolved. In Mehra and Bloom's work, in which they observed adjuvant activity of cord factor on *M. leprae* in guinea pigs, they

TABLE 2. Results of experiment 3, testing Talwar's culture MW (and the adjuvant effect of aqueous cord factor on *M. leprae* [ML] and the efficacy of the subcutaneous route for ML)^a

Group	Vaccine	Reaction to vaccine				Corrected FPE (48 h)			Protection			
		Site, 28 days		Ln, 28 days		Ag ^{c,d}	0.01 mm	P ^b	6 mo		9 mo	
		mm	P ^b	mm	P ^b				Log ₁₀	P ^b	Log ₁₀	P ^b
C	MW	1.86 ^e	<0.0002	3.80 ^e	0.0002	ML	-4	NS	0.16	NS	0.32	NS
D	MW ^d	1.87	<0.0002	3.91 ^f	0.0026	ML	15	NS	0.15	NS	0.29	NS
F	ML ^d	1.40 ^g	<0.0002	4.01 ^g	0.0012	ML	11.5	NS	0.31	NS	1.08	<0.002
H	BCG	2.26 ^h	<0.0002	5.09 ^h	<0.0002	ML	59.5	<0.01	>2.05	<0.002	2.27	<0.002
I	ML ^d + CF ⁱ	1.05	<0.0002	3.75	0.02	ML	-4	NS	0.44	NS	1.20	<0.002
J	ML, ^d s.c. ^j	0.00	NS	3.30	NS	ML	-2.5	NS	0.37	NS	0.60	<0.02
L	Diluent	0.00 ^k		3.09 ^k		ML	(35.5) ^l		(5.85) ^m		(6.20) ^m	
A	MW					MW	100	<0.002				
B	MW ^d					MW	97	<0.002				
E	ML ^d					MW	35	<0.05				
G	BCG					MW	117.5	<0.002				
K	Dil					MW	(92.5) ^l					

^a The reaction at the vaccination site and the size of the regional lymph node (Ln) are shown at 28 days. The corrected FPE is given for 48 h after challenge at 28 days with 10⁷ heat-killed mycobacteria. Vaccine protection [log (average harvest in controls) - log (average harvest in vaccinated group)] is shown at 6 and 9 months after footpad challenge at 28 days with 5,000 *M. leprae* (viable suspension).

^b Versus control groups. NS = *P* > 0.10 (two-tailed test values).

^c Ag, Challenge antigen.

^d Heated to 100°C for 30 min.

^e Groups C and A.

^f Groups D and B.

^g Groups F and E.

^h Groups H and G.

ⁱ CF, Cord factor.

^j s.c., Subcutaneous.

^k Groups L and K.

^l Uncorrected value.

^m Average harvest in controls.

emulsified the cord factor in aqueous media (6). Therefore, in the present experiment for group I, the cord factor was in aqueous suspension (suspended by ultrasonication and then mixed with a suspension of *M. leprae*). The results again showed no adjuvant effect of cord factor (group I versus group F). For group J, the *M. leprae* vaccine was injected subcutaneously because an earlier experiment (Shepard et al., submitted) had shown an *M. leprae* suspension to be immunogenic subcutaneously, as judged by FPE. Experiments before that (13) had indicated the subcutaneous route to be less effective. The present results show the subcutaneous route to be effective, and not significantly less so than the intradermal route (group J versus group F).

DISCUSSION

Mycobacteria share many antigens, and at the start it was not clear whether the mere sharing of determinants (at least those to which the mice could be sensitized) was important or whether the sharing of a particular determinant(s) was necessary for protection against infectious challenge. For example, many of the tests from mycobacterial antigens (whether by serological

means or by delayed hypersensitivity tests) emphasize protoplasmic antigens or antigens released in a soluble state from old cultures; instead, one or more integral surface antigens might be critical for vaccine protection. The results of experiment 1 were not informative in this regard because none of the cultures except BCG was found to be strongly immunogenic even for homologous antigen(s), as judged by local reactions and regional lymph node enlargement (and none of the cultures except BCG provided highly significant protection against *M. leprae* infection).

In experiments 2 and 3, however, several cultures were found to produce local reactions and regional lymph node enlargement. These were *Mycobacterium* sp. 100616, *M. tuberculosis* H37Ra, the two strains of *M. simiae*, *M. vaccae*, and MW (in addition to BCG). Such reactions could have been caused by locally persisting infections rather than immunization, but *Mycobacterium* sp. 100616- and MW-vaccinated mice showed corrected FPE when challenged with homologous antigen. More importantly, mice immunized with *Mycobacterium* sp. 100616, *M. tuberculosis* H37Ra, the two strains of *M. simiae*, and *M. nonchromogenicum* manifested sig-

nificant corrected FPE on challenge with *M. leprae* antigen. Thus, even though these cultures appeared to sensitize the mice to *M. leprae* determinants, they did not afford protection against *M. leprae* infectious challenge.

It might be argued that because the sensitization produced by these cultures to *M. leprae* antigens in FPE tests was a lesser degree, it would be insufficient to provide protection against infectious challenge. The results of unpublished work show, however, that a low dose of *M. leprae* can provide good protection against infectious challenge without generating sufficient sensitization to produce detectable corrected FPE.

Thus, BCG differed from the other cultures in providing good protection against *M. leprae* infectious challenge and in sensitizing to produce good FPE when challenged with heat-killed *M. leprae*. These results appear to indicate that BCG differs from the other cultures in possessing an antigen critical to protection against *M. leprae*. Another possibility is that the prominent adjuvant activity of BCG causes it to sensitize the mice to a broader range of the antigenic determinants it contains.

BCG affords protection against many experimental mycobacterial infections (reviewed in 13) and even against certain tumors. The antitumor activity was widely interpreted as evidence that BCG has a nonspecific effect, presumably by local macrophage activation or by adjuvant activity on tumor antigens, but even tumors may share antigenic determinants with BCG (2, 7). Thus, although the present work emphasizes the uniqueness of BCG among mycobacteria in providing protection against *M. leprae*, it does not provide the evidence needed to choose among the possible mechanisms for this activity.

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